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RESEARCH ARTICLE

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An insight into the diagnostic and prognostic value of HOX A13's expression in non-muscle invasive bladder cancer

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Abstract

Background: Several studies have interrogated the molecular pathways and their interacting genes underlying bladder cancer (BCa) tumorigenesis, yet, the role of homeobox genes is still poorly understood. Specifically, HOXA13, which plays an important role as a major actor in the urogenital tract's development.

Methods: Immunohistochemical (IHC) staining was performed to inspect the differential expression of HOXA13 protein in non-muscle-invasive bladder cancer (NMIBC) and non-tumoral tissues. A semiquantitative scoring system was adopted to evaluate the IHC labeling. Correlation to clinical parameters was performed by descriptive statistics. Overall survival was estimated by the Kaplan–Meier method and Cox regression model. The functional HOX A13 protein association networks (PPI) were obtained using String 11.0 database.

Results: HOX A13 exhibited cytoplasmic and nuclear staining. Its expression levels were lower in high-grade NMIBC (HG NMIBC) compared to low-grade ones (LG NMIBC). The expression of HOX A13 was correlated to tumor grade (LG/HG) ($p = 0.036$) and stage (TA/T1) ($p = 0.036$). Nevertheless, its expression was not correlated to clinical parameters and was not able to predict the overall survival of patients with HG NMIBC. Finally, PPI analysis revealed that HOX A13 seems to be a part of a molecular network holding mainly PBX1, MEIS, ALDH1A2, HOX A10, and HOX A11.

Conclusion: The deregulation of HOX A13 is not associated with the prognosis of BCa. It seems to be rather implicated in the early initiation of urothelial tumorigenesis and thus may serve as a diagnostic marker in patients with NMIBC. Further experimentations on larger validation sets are mandatory.

KEYWORDS
bladder cancer, diagnosis, HOXA13, PPI, prognosis

Giulia Piaggio and Slah Ouerhani contributed equally to this work.

1 | BACKGROUND

With 430,000 cases diagnosed throughout the world,[1] urothelial carcinoma of the bladder represents one of the most frequent genitourinary malignancies in the world.[2] In Tunisia, it lines up in the first rank among urological cancers in men.[3] Thus, research on bladder cancer (BCa) represents the main challenge in the local oncology arena. Approximately 70% of newly diagnosed cases are non-muscle-invasive bladder cancer (NMIBC). It is a heterogenous population of tumors englobing a significant proportion of T1 tumors (70%) (invasion of the subepithelial connective tissue or the lamina propria), Ta tumors (20%) (confined to the epithelium or mucosa), and carcinoma in situ (CIS) (10%) (flat, high-grade, non-papillary carcinomas confined to the urothelium).[4]

Non-muscle invasive tumors usually display a favorable prognosis despite their elevated recurrence rate (40%–80%). Nevertheless, nearly 15% of NMIBC patients will experience progression to muscle-invasive and/or metastatic disease associated with an unfavorable prognosis.[5] The 5-year progression rates of low-risk NMIBC vary from 0.8% to 6%, whereas 30% of patients with high-risk NMIBC will develop progressive disease.[6] Therefore, the prediction of tumor progression is of a major importance for setting up individual prognosis and appropriate follow-up strategy. Actually, tumor grade is the most important factor for predicting a poor clinical outcome in NMIBC.[7] Although, the prognosis spectrum based on clinicopathological parameters seems insufficient in accurately predicting the prognosis and managing NMIBC patients with diverse and complicated tumor backgrounds. Thus, additional prognostic indicators, used alone or in combination with other clinicopathological parameters, are needed to tailor more accurate surveillance and management.

By referring to recent data, it seems obvious that it is necessary to not only consider clinical and pathological factors but also take into account the potential effect of molecular alterations present in the tumors when considering NMIBC's prognosis.[8]

In this context, a major focus has been nowadays attributed to research investigations evolving *HOX* genes (Homeobox genes) in cancer because of their critical roles in mastering embryonic development process. Moreover, it has been recently proven that dysregulated expression of *HOX* genes is closely linked to tumor development and progression of genitourinary neoplasms, mainly bladder and prostate cancers,[9] as they may influence a large number of hallmarks of cancer's pathways that are crucial for proliferation and maintenance during tumor growth.[10] In this regard, their deregulation has been reported to enhance cell proliferation, invasion or metastasis, or to be associated with drug resistance processes and BCa prognosis as it was positively correlated to lymph nodes metastases, TNM stage, pathological grade, and patient survival.[11-14] Besides, few reports describe the prognostic value of the cytoplasmic delocalization and the methylation status of HOX genes in BCa.[15,16]

Specifically, HOX A13 is located in the most posterior cluster of the HOX members (HOX 13 genes). It is expressed in the genital tubercle during embryogenesis and thus described to play an important role in the development of the external genital and urogenital tract.[11] Although the abnormal expression of HOX families in BCa tissues suggests that they might play a role in the development of BCa, yet, the correlation between HOXA13 expression and prognosis in NMIBC has not been well reported.

In this context, our main focus in the present research is to uncover the expression of HOX A13 in patients with urinary NMIBC, and appraise the clinical impact of its potential deregulation.

2 | MATERIAL AND METHODS

2.1 | Samples collection and ethics statement

This retrospective study was accomplished on formalin-fixed paraffin-embedded (FFPE) tissue samples obtained from the Anatomy Pathology Department from patients who had undergone transurethral resection of the bladder (TURBT) at the Urology Department of Charles Nicolle Hospital (Tunis, Tunisia).

This study conformed to the provision of Helsinki's Declaration and was agreed to by the Charles Nicolle's Ethics Committee (approved on 17-03-2016). An individual written informed consent form was obtained from all patients.

2.2 | Patients enrollment

Specimens numbering 42 belonging to patients with NMIBC were recruited for the present investigation as 25 high-grade (HG) NMIBC samples and 17 low-grade (LG) NMIBC ones. Among several paracancerous tissues obtained from the non-tumoral zones of cystectomy-resected specimens', two samples were selected for the analysis as non-tumoral controls as they were histomorphologically and cytomorphologically confirmed to be non-tumoral in order to ensure an accurate interpretation of the data.

Tissues were acquired based on the histopathological evaluation. NMIBC tumors were first identified by cystoscopy and further confirmed by subsequent TURBT. Tumor stage was established according to the TNM classification along with the cross-sectional imaging and the pathological evaluation of the TURBT as pTa and pT1 in accordance with the invasion of the lamina propria.

Histological grades were settled according to the 2004/2016 World Health Organization (WHO) guidelines as LG tumors or HG tumors according to cellular atypia and alterations in tumor architecture.

Experimented pathologists, who were blinded to the clinical data, appraised the staining results. In general, an interobserver disagreement rate was irrelevant. Discrepancies were dispatched by consensus after re-evaluating inconclusive cases or through consulting a third pathologist when agreement could not be reached.

Baseline clinical data and tumor characteristics were reacquired from patients' charts and pathological reports. The specimens involved in the current research were primary tumors. Recurrent

patients and those with initially pT1 tumors that progress to the muscle-invasive form of BCa were not counted. Patients with concomitant histologies other than urothelial carcinoma of the bladder, or formerly treated with chemotherapy and/or radiotherapy, were excluded. The patient's background and clinicopathological features are recapitulated in Table 1.

Patients with a NMIBC were stratified into risk groups in accordance with the prediction model of the European Organization for Research and Treatment of Cancer (EORTC) in an attempt to enhance the predictive accuracy. This classification supplies a scoring system for assessing clinical outcomes in terms of recurrence and progression, and establishes recurrence and progression rates of NMIBC patients at 1 and 5 years (Table 2).

TABLE 1 Demographic and clinicopathological features of the studied population

Characteristics	Value
Samples size	42
Mean age (years)	71 (35–95)
Gender	
Male	40/42 (95.2%)
Female	02/42 (04.8%)
Tumor grade	
LG NMIBC	17/42 (40.5%)
HG NMIBC	25/42 (59.5%)
Tumor stage	
Ta	17/42 (40.5%)
T1	25/42 (59.5%)
Tumor multifocality	
No	20/42 (47.6%)
Yes	21/42 (50%)
Unavailable data	01/42 (2.4%)
Tumor size	
<2 cm	12/42 (28.6%)
≥2 cm	28/42 (66.6%)
Unavailable data	02/42 (4.8%)

Abbreviations: HG NMIBC, High-Grade Non-Muscle-Invasive Bladder Cancer; LG NMIBC, Low-Grade Non-Muscle-Invasive Bladder Cancer.

The recurrence and progression rates of each group were concluded with the EORTC risk tables (http://www.eortc.be/tools/bladdercalculator/).

2.3 | Qualitative and descriptive evaluation of staining quality

Immunohistochemical staining (IHC) was performed on 4-µm-thick sections of paraffin-embedded surgically resected tissues. Anti-HOX A13 antibody [1:200; PH = 9] (Thermofisher Scientific, PA5-40459) was selected and used in immunohistochemical staining. A secondary staining detection (Leica Biosystems) was performed following the manufacturer's instructions, and protein staining was analyzed under optical microscopy. A positive IHC staining was set as yellow-brown color according to the manufacturer's demonstrative slides.

We set a semiquantitative scoring system based on the coalition of the expansion of positively stained tumor cells (Score [1]: 0: <1%, 1: 1%–30%, 2: 30%–70% and 3: >70%) and the intensity of staining reaction (score [2]: 0 = absent; 1 = weak; 2 = moderate; 3 = strong). The final immunostaining score was the product of score 1 multiplied by score 2 in each individual slide. Finally, we explicated final immunostaining scores as follows: scores 0, 1, 2 and 3 as low expression, and scores 4, 6 and 9 as high expression.[11]

2.4 | Survival analysis

The patients recruited for the present analysis must have at least one complete follow-up schedule indicating their living status for a while. The median follow-up was set for 36 months and the overall survival (OS) was settled as the time from the date of the primary treatment to the date of the last clinical check-up. Indeed, 13 LG NMIBC and 20 HG NMIBC patients were considered for the survival analysis. Patients in both tumor groups were stratified according to "time to death", as it is the event of interest, and to the level of expression of HOX A13 (High, Low) (Table 3).

For the analysis, we used SPSS 25.0 software (SPSS Inc.) in order to analyze the data. The correlation between overall survival (OS)

TABLE 2 Prediction of disease recurrence and progression in patients with NMIBC according to the EORTC prognostic scoring system

Clinical parameters	Score	Probability of recurrence or progression at 1 year (%)	Probability of recurrence or progression at 5 years (%)	Recurrence or progression risk groups	Average
Recurrence	5–9	38 (35–41)	62 (58–65)	Intermediate risk	33/42 (78.6%)
	10–17	61 (55–67)	78 (73–84)	High risk	06/42 (14.3%)
				Unavailable data	03/42 (07.1%)
Progression	2–6	1 (0.4–1.6)	6 (5–8)	Intermediate risk	11/42 (26.2%)
	7–13	5 (4–7)	17 (14–20)	High risk	28/42 (66.7%)
				Unavailable data	03/42 (07.1%)

Abbreviations: EORTC, European Organization of Research and Treatment of Cancer; NMIBC, Non-Muscle-Invasive Bladder Cancer.

TABLE 3 Patient data and stratification according to HOX A13's expression for the OS analysis

	Mortality		HOX A13 expression	
Patients	Yes	No	Low	High
LG NMIBC ($N = 13$)	02/13 (15.38%)	11/13 (84.62%)	07/13 (53.85%)	06/13 (46.15%)
HG NMIBC ($N = 20$)	09/20 (45%)	11/20 (55%)	17/20 (85%)	03/20 (15%)

Abbreviation: OS, Overall Survival.

and HOX A13's expression was evaluated using the Kaplan–Meier method. The Log-Rank test was applied to compare survival curves. Finally, data were analyzed by adjusting a Cox proportional hazard regression model in order to appraise the joint effects of the analyzed covariates on patient's survival. The pathological variables included were age at diagnosis of tumor stage, tumor size, number of tumor foci, and EORTC progression score in addition to the expression status of HOX A13.

2.5 | Statistical analysis

Descriptive analyses were performed using the Statistical Package for the Social Sciences, version 25.0 (SPSS Inc.). Chi-square test, Mann Whitney *U* and Pearson's correlation were adopted for the analyses. A *p*-value <0.05 was considered statistically significant.

2.6 | Functional protein association networks

The functional protein association networks were obtained using the String 11.0 database (https://string-db.org/). It is a biological resource of known and predicted protein–protein interactions (PPI). It implements well-known classification systems such as Gene Ontology (GO) and KEGG, but also offers additional, new classification systems based on high-throughput text mining as well as on hierarchical clustering of the association network itself.[17]

3 | RESULTS

3.1 | Clinicopathological data

A total of 42 patients with urinary NMIBC were included. They were composed of 40 males and 2 females, with ages ranging from 35 to 95 years (mean 71 years). The median follow-up after the TURBT was 36 months (range, 0–148 months); 40.5% of the tumors were low grade, whereas 59.5% were high grades. Pathological stage Ta was observed in 40.5% of cases and T1 in 59.5%; 47.6% of tumors rose from a unique site, whereas 50% of them were multifocal. Detailed clinicopathological descriptions of the included patients is summarized in Table 1.

3.2 | HOX A13 is expressed in the majority of bladder specimens and is located in both the cytoplasm and the nucleus

The analysis of HOX A13 staining revealed a homogenous nuclear expression in non-tumoral controls (nuclear expression, IHC score = 4, high expression) and heterogeneity of expression in patients with NMIBC ($p = 0.03$) in terms of location, intensity, and labeling score (Figure 1).

Indeed, HOX A13 expression was assessed within both subgroups of NMIBC (Table 4) ($p = 0.04$). As a result, nuclear expression of the targeted protein was observed in only 11.1% of LG NMIBC samples, whereas it was cytoplasmic in patients with HG NMIBC (Figure 1). A low expression of HOX A13 was detected in 34.4% of LG NMIBC and 70% of HG NMIBC (Figure 2A) while a high expression was uncovered in 65.6% of LG NMIBC and 30% of HG NMIBC (Figure 2B).

3.3 | HOX A13 expression is associated with Ta/T1 stage and histological grade

Immunohistochemical scores of HOX A13 were correlated with clinical prognostic parameters namely gender, age, tumor stage Ta/T1, tumor grade (LG vs. HG), tumor multifocality, and tumor size in NMIBC patients (Table 5). Interestingly, a significant association was perceived between HOX A13 staining score (expression) and tumor grade (LG/HG) ($p = 0.036$) and stage (Ta/T1) ($p = 0.036$) but not with the other pathological characteristics. Besides, immunostaining scores related to HOX A13's protein expression were not related to EORTC progression and recurrence scores.

3.4 | Association of HOXA13 protein with survival

The potential implication of deregulated HOX A13's protein expression on overall survival was cross-examined in low-grade and high-grade NMIBC patients. Unfortunately, no significant association was revealed in our study either in the LG NMIBC group (Figure 3A) or in the HG NMIBC group (Figure 3B) ($p = 0.713$, $p = 0.281$, respectively).

On its side, Cox regression analysis did not come with a statistically significant influence of the analyzed pathological variables and HOX A13 expression status on overall survival ($p > 0.05$).

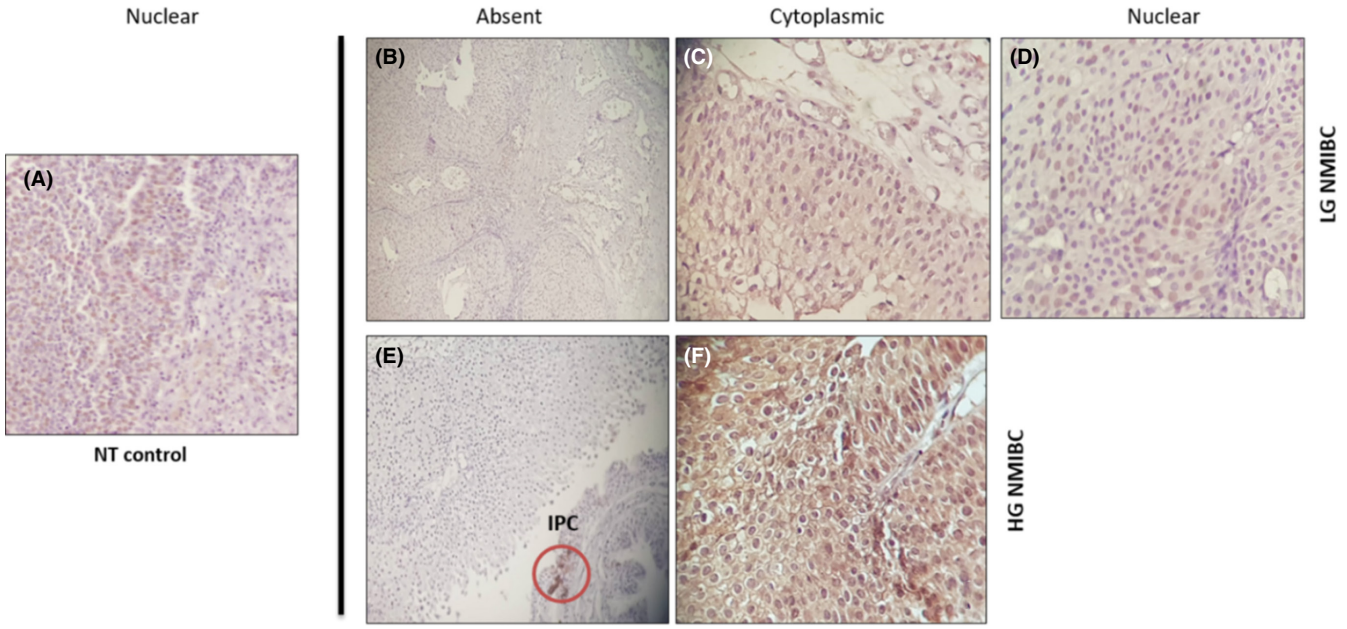

FIGURE 1 The protein expression of HOX A13 in urothelial NMIBC tissues. (A, B, E) (X10), (C, D, F) (X40); NMIBC, Non-Muscle Invasive Bladder Cancer; LG NMIBC, Low-Grade Non-Muscle Invasive Bladder Cancer; HG NMIBC, High-Grade Non-Muscle Invasive Bladder Cancer; NT, Non-tumoral; IPC, Internal Positive Control; IHC, immunohistochemistry). (A) Positive nuclear immunostaining of HOX A13 in non-tumoral urothelial tissue NT (cell labeling intensity score 2; 50% of labeled cells; IHC score 4). (B) Absent immunostaining of HOX A13 in low-grade non-muscle invasive tissue. (C) Positive Cytoplasmic immunostaining of HOX A13 in low-grade non-muscle invasive tissue (cell labeling intensity 1, 80% of labeled cells, IHC score 3). (D) Negative nuclear immunostaining of HOX A13 in low-grade non-muscle invasive tissue (cell labeling intensity 1, 1% of labeled cells, IHC score 0). (E) Absent immunostaining of HOX A13 in high-grade non-muscle invasive tissue (IPC: lymphocyte cells). (F) Positive Cytoplasmic immunostaining of HOX A13 in high-grade non-muscle invasive tissue (cell labeling intensity 1, 50% of labeled cells, IHC score 2)

TABLE 4 The expression of HOX A13's protein in patients with NMIBC

Positive stained cells			Staining intensity			Final staining score (IHC score) (score1*score 2)		
Percent (%)	Score 1	*N* (%)		Score 2	*N* (%)	Score 3	*N* (%)	Expression
<1%	0	45.2	Absent	0	45.2	0–1	64.3	Low
1%–30%	1	28.6	Weak	1	21.4	2–3	12.0	
30%–70%	2	21.4	Moderate	2	26.2	4–6	19.0	High
>70%	3	04.8	Strong	3	07.2	9	04.7	

3.5 | HOX A13 protein-interactions (PPI) analysis

By performing the PPI analysis, we were able to single out predicted and/or validated proteins that interact with *HOX A13* during the tumorigenesis process (Figure 4). Among these, *HOX A13* seems to be a part of a molecular network holding mainly *PBX1*, *MEIS1*, *MEIS2*, *MEIS3*, *ALDH1A2*, *HOX A10*, and *HOX A11* (Figure 4, Table S1).

4 | DISCUSSION

Genes involved in the regulation of normal cell proliferation and differentiation, contribute to the tumorigenesis' enrollment and/or tumor progression due to their deregulated function or to their interaction with deregulated target genes. While many studies have investigated the molecular pathways underlying BCa development, yet, the role of homeobox genes is still poorly understood. These genes encode regulatory proteins. Among these, the homeobox A cluster gene family (HOXA), specifically HOXA13, a member of HOX13 paralogous genes, are involved in controlling the spatial expression patterns of target genes[1,2,18,19] and in mediating urogenital system formation.[20-22] However, many of them are still active in adult human organs and tissues[23] and are frequently deregulated in human cancers.[15,23-25] Recently, some studies reported the detection of HOXA13's in the urine of patients with BCa[26,27]. Yet, there are few studies on the expression profile and prognostic values of these genes in BCa tissue specimens.[11] In the present study, we have enrolled the investigation of HOX A13 protein's expression in

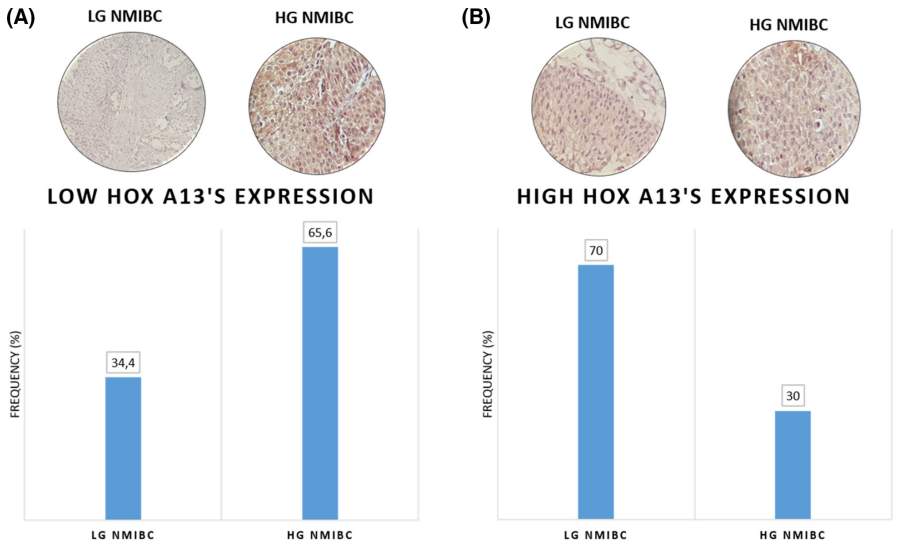

FIGURE 2 HOX A13 expression according to tumor groups. (A) Low expression of HOX A13 in LG NMIBC and HG NMIBC. (B) High expression of HOX A13 in LG NMIBC and HG NMIBC. NMIBC, Non-Muscle Invasive Bladder Cancer; LG NMIBC, Low-Grade Non-Muscle Invasive Bladder Cancer; HG NMIBC, High-Grade Non-Muscle Invasive Bladder Cancer; IHC, immunohistochemistry). The frequencies were calculated using the frequency test in the descriptive statistics provided by IBM SPSS statistics 26 package

TABLE 5 Correlation between IHC score related to HOX A13's protein expression and clinicopathological prognostic factors

		HOX A13 expression		
		Low	High	
Clinical parameters		***N* = 31**	***N* = 11**	***p*** values*
Age (years)	<71	17	02	0.823
	≥71	14	08	
	ND	-	1	
Gender	M	29	11	0.576
	F	02	00	
Tumor stage	Ta	10	07	**0.036**
	T1	21	04	
Tumor grade	LG	10	07	**0.036**
	HG	21	04	
Tumor size	<2 cm	08	02	0.657
	≥2 cm	22	08	
	ND	02	-	
Tumor multifocality	1	14	05	0.152
	≥2	16	06	
	ND	01	-	
EORTC recurrence scores	Intermediate	25	08	0.685
	High	05	01	
	ND	02	01	
EORTC progression scores	Intermediate	07	04	0.438
	High	22	05	
	ND	02	01	

Note: *p** value for khi^2 test, Mann Whitney *U* test or Pearson correlation test; Significant *p* values are in Bold; IHC (immunohistochemistry) staining scores were established according to a semiquantitative analysis.

Abbreviations: EORTC, European Organization of Research and Treatment of Cancer; F, Female; HG, High grade; LG, Low grade; M, Male; ND, non-available data.

patients with NMIBC. It exhibits a high nuclear expression in non-tumoral controls and a negative or low expression in patients with NMIBC ($p = 0.03$) suggesting a tumor-suppressive role of HOX A13. This result is in disagreement with the one suggested by the unique work uncovering the expression of HOX A13 in bladder tumors and which reported rather a high cytoplasmic expression of HOX A13

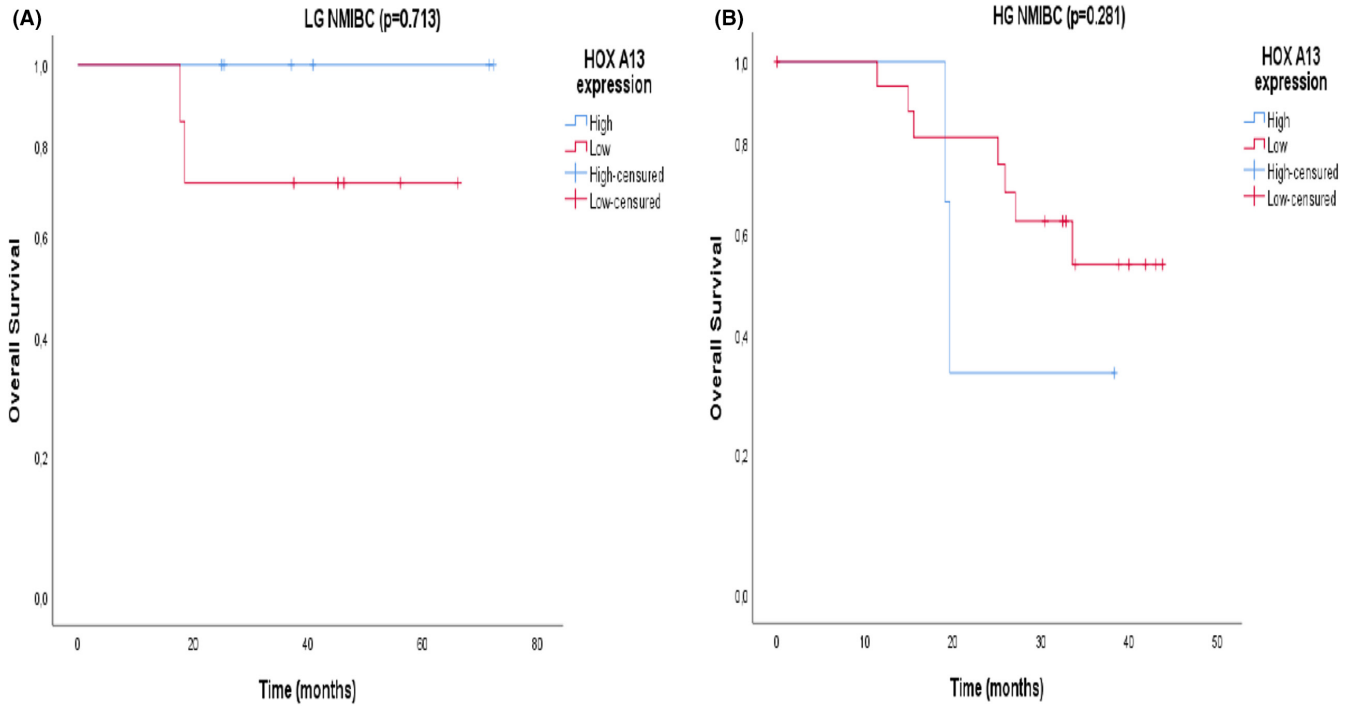

FIGURE 3 Kaplan–Meier survival curves of overall survival (OS) according to the expression levels of HOX A13. NMIBC: Non-Muscle Invasive Bladder Cancer; IHC, immunohistochemistry; *p* value for Kaplan Meier Analysis

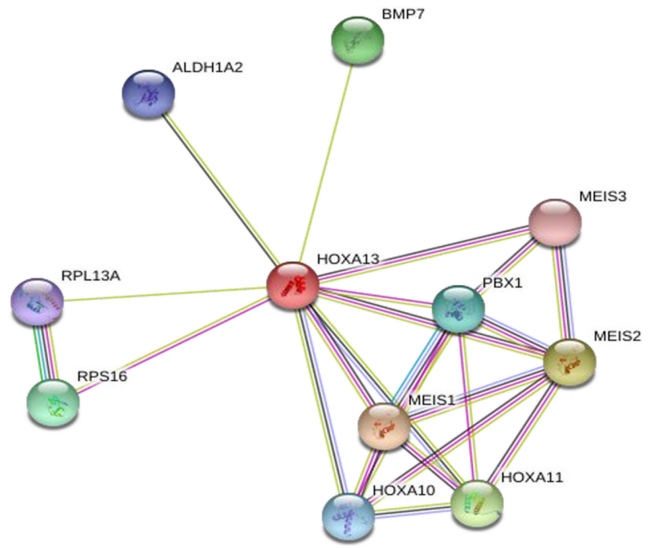

FIGURE 4 Functional HOX A13 PPI interactions networks obtained using String 11.0 database. Network nodes represent proteins: splice isoforms or post-translational modifications are collapsed; i.e., each node represents all the proteins produced by a single protein-coding gene locus. The contributing nodes are classified regarding degree values. Edges represent protein–protein associations

compared with controls.[11] The divergence in terms of high and low expression could be explained by the fact that in our investigation, we included only NMIBC in the opposite of the study conducted by Hu et al. in which both NMIBC and muscle-invasive bladder cancer patients were involved, or to the fact that their cohort of study was larger. Nevertheless, both studies clearly spotlight the potential diagnostic role of this marker in patients with urothelial bladder carcinoma.

Besides, when comparison was based on IHC score, *HOX A13* was significantly and differentially expressed between LG NMIBC and HG NMIBC ($p = 0.036$) which is in agreement with a preceding work.[11] By taking the localization as a benchmark, *HOX A13* was predominantly expressed in the cytoplasm in LG forms (11.1% of cases show nuclear expression against 44.4% having cytoplasmic expression), and entirely cytoplasmic in patients with HG NMIBC. This double location, nuclear and cytoplasmic, could be explained by the modulation of nuclear localization signals causing the export of *HOX A13* outside the nucleus [28,29]. These findings have been described in a previous study on gastric tumors [30] and prostate cancer [31], and are in contradiction with the one reported on BCa.[11] In addition, our results add a new insight into the role of *HOX A13* deregulation in BCa and indicate clearly that it could help pathologists distinguishing LG and HG tumors especially in tumors with a conflictual background.

On the other hand, the alliance of HOX *A13's* expression on clinical features pinpoints the fact that it was not altered due to the clinical presentation of the tumor, and could predict neither recurrence nor progression in patients with NMIBC. These data supposed that HOX A13 deregulation could be the consequence of the interference of numerous other factors referring to genetic or epigenetic fields, and that it is involved in tumorigenesis' initiation rather than prognosis.

The investigation of the overall survival in NMIBC patients with regard to the expression patterns of HOX A13 was further questioned. Although, this result was statistically irrelevant (Figure 3A,B)

($p = 0.713$, $p = 0.281$) which is in disagreement with preceding data regarding HOX A13.[11,18,20] This could be explained by the retrospective nature of the present study in addition to the relatively small cohort of patients with NMIBC.

To sum up, our data confirm the diagnostic value of HOX A13 for patients with NMIBC and strongly suggest its potential tumor-suppressing role. To accomplish this task, these proteins may interact with other proteins such as *PBX1* regulatory protein-1, *MEIS* which are known as cofactors for HOX-class homeobox proteins, competing functionally with each other[32] and binding to DNA as heterodimer or trimeric structures along with HOX genes, and thus directly regulating tumor progression.[33-35] In addition to HOX A10 and HOX A11 that were reported to be involved in hallmark pathways known to be implicated in urothelial tumorigenesis' initiation and/or progression such as ERK, TGFβ2-p38 MAPK or PI3K/AKT signaling pathways.[36,37,39] These speculations were derived through the combination of in silico findings with the literature data. Functional studies are mostly advocated to support the reported data and to uncover the molecular mechanism of tumor initiation and/or progression driven by *HOX A13* in urinary BCa

5 | CONCLUSION

Our results suggest the involvement of *HOX A13* protein in the early initiation of urothelial carcinogenesis. This marker seems to be roughly implicated in BCa diagnosis and could be considered as potential diagnostic biomarkers for patients with NMIBC.

AUTHOR CONTRIBUTION

Conceptualization, Guilia PIAGGIO and Slah OUERHANI; data curation, Nouha SETTI BOUBAKER, Aymone GURTNER, Nesrine TRABELSI, Isabella MANNI; formal analysis: Nouha SETTI BOUBAKER, Aymone GURTNER, Nesrine TRABELSI, Isabella MANNI, Ahlem BLEL and Slah OUERHANI; funding acquisition: none; investigation, Nouha SETTI BOUBAKER, Haroun AYED; methodology, Nouha SETTI BOUBAKER, Aymone GURTNER, Ahlem BLEL; software: Nouha SETTI BOUBAKER, Nesrine TRABELSI; statistical analysis, Nouha SETTI BOUBAKER; project administration, Haroun AYED, Guilia PIAGGIO and Slah OUERHANI; resources, Haroun AYED, Ahlem BLEL, Ahmed SAADI,, Marouene CHAKROUN, Zeineb NAIMI, Selim ZAGHBIB, Meriam KSONTINI, Khedija MEDDEB, Soumaya RAMMEH, Mohamed CHEBIL, Guilia PIAGGIO and Slah OUERHANI; supervision, Slah OUERHANI; validation:, Aymone GURTNER, Guilia PIAGGIO and Slah OUERHANI; visualization, Nouha SETTI BOUBAKER; writing – original draft,, Nouha SETTI BOUBAKER; writing – review & editing, Haroun AYED, Guilia PIAGGIO and Slah OUERHANI. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare there are no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

PATIENT CONSENT STATEMENT

All the patients recruited as a part of any research or experiment described in this study have given written consent to the inclusion of material pertaining to themselves. Their identities were fully anonymized.

CONSENT FOR PUBLICATION

Additional informed consent was obtained from all participants.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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